

Target amplification systems in nucleic acid-based diagnostic approaches

BY DEBORAH Y. KWOH AND T. JESSE KWOH

BEFORE THE ADVENT of in vitro nucleic acid amplification systems, the application of nucleic acid hybridization in clinical diagnosis for infectious and genetic diseases had long been problematical. The major impediment in developing nucleic acid-based diagnostic systems has been sensitivity of the detection method. Rapid, nonisotopic, nucleic acid-based diagnostic systems could only be developed for target sequences which were highly abundant. Detection of rare sequences required the use of long assays, highly radioactive probes, and large tissue samples, because the most sensitive hybridization probes, those continuously labeled with ^{32}P , can detect a sequence only when at least 10^4 to 10^5 molecules of the target are present in a sample.

The major breakthrough for the use of nucleic acid hybridization in routine clinical diagnosis was the development of the polymerase chain reaction (PCR).^{1,2} With PCR, rare sequences in minute tissue samples could now be amplified 10^6 -fold or more in several hours, which enabled the routine use of nonisotopic detection systems and oligonucleotide probes for rapid hybridization-based diagnostic assays. Since the first report describing PCR appeared in 1985, a multitude of modifications to the basic PCR protocol have appeared extending the utility of the procedure to a diverse range of applications in disease diagnosis, forensic sciences, and basic biological research (see Refs. 2 and 3 for reviews).

The advent of PCR also stimulated the development of several alternative in vitro nucleic acid amplification methods such as the transcription-based amplification system (TAS),⁴ the self-sustained sequence replication (3SR) system,⁵ the ligation amplification reaction (LAR)⁶ or ligase-based amplification system (LAS),⁷ and an RNA replication system based on the Q β replicase.⁸ This article will review these amplification protocols and compare their features.

Amplification by DNA synthesis (PCR)

The PCR amplification system developed by K. Mullis and co-workers^{1,2} is elegantly simple in concept yet tremendously powerful in application. Basically, the method involves repeated cycles of the DNA polymerase-mediated primer extension reaction (Figure

1). DNA synthesis of the target sequence is directed by two oligonucleotides which bracket the target sequence on opposite strands of the DNA. By using a thermostable DNA polymerase, the reaction can be repeatedly cycled through alternating thermal DNA denaturation, primer hybridization, and primer extension reaction steps in a single tube until sufficient amplification of the target sequence has been achieved. Typically, a 10^6 -fold amplification of the target can be achieved in 2-4 hr through 30 thermal cycles (Table 1).

The use of PCR for target sequence amplification has several advantages (Table 2a). The reaction uses a single thermostable enzyme and can be performed in a single tube without repeated reagent addition. Thermal

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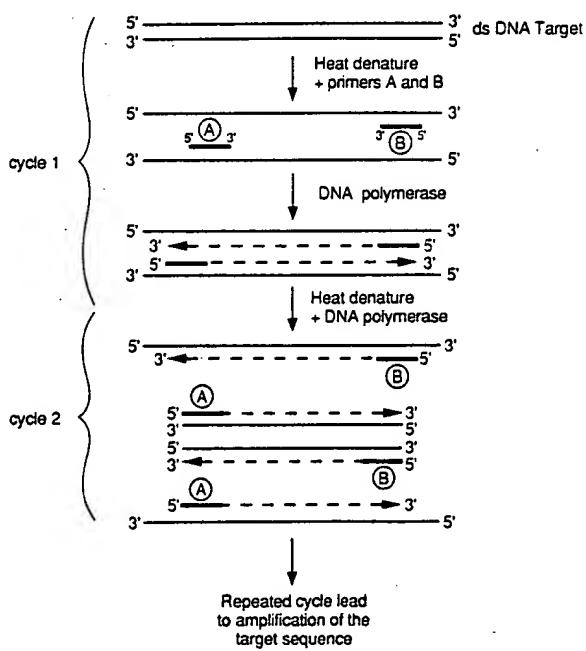


Figure 1 PCR—a double-stranded (ds) DNA target is thermally denatured in the presence of primers A and B, which are complementary to opposite strands of the target DNA. After the primers are allowed to anneal to the denatured DNA strands, a DNA polymerase extends the primers using the target DNA strands as templates. In the second cycle, the newly synthesized DNA is thermally denatured from the target DNA strands. Primers A and B are again annealed to the appropriate DNA strands and the DNA polymerase extends the primers. Repeated cycles of denaturation and DNA synthesis produce an exponential replication of a segment of the target DNA molecule.

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Table 1

Performance characteristics of different amplification approaches

	PCR	3SR	LAR/LAS	Qβ
Reaction type	DNA synthesis	RNA transcription	DNA ligation	RNA replication
Amplification				
Amount	10 ⁶ -fold ^a	10 ⁶ -to 10 ⁹ -fold ^b	40-fold ^c	(10 ⁶ -to 10 ⁹ -fold) ^d
Time required	2.5 hr ^e	1 hr	4 hr	(1 hr) ^d
Number of cycles	30	1	10	1
Denaturation steps	30	0 if RNA 2 if DNA	10	1 if RNA 2 if DNA
Product				
Size	100 bp-10 kbp	200-600 bases	30-60 bp	250-300 bases
Type	ds ^f DNA	ss ^g RNA (+ RNA:DNA + (ds DNA))	ds DNA	ss RNA
Enzymes required	Taq DNA polymerase	AMV reverse transcriptase, T7 RNA polymerase, RNase H	DNA ligase	Qβ replicase, reverse transcriptase, T7 RNA polymerase

^a For amplification of 200 base segment of HIV RNA (Ref. 9).^b For amplification of 200 base segment of HIV RNA (Ref. 5 and unpublished results).^c From Ref. 7.^d Estimated based on Ref. 4 for TAS portion and Ref. 8 for Qβ portion of protocol.^e Time for manual temperature cycling (Ref. 9). Processing using automated thermal cycling machine requires 4 hr.^f Double-stranded.^g Single-stranded.

cycling of the reaction can be easily automated through the use of commercially available programmable temperature cycling devices. Further, amplification of RNA target sequences can easily be accomplished by synthesis of a cDNA with reverse transcriptase prior to PCR. Another advantage of PCR is that the reaction product usually has a discrete size so that the success of the reaction can often be judged by the mobility of the product in gel electrophoresis.

However, PCR does suffer from several problems (Table 2b). Among these problems is the number of steps involved in the thermal cycling of the reaction. For 30 cycles of PCR, 90 temperature transfers must be performed and the incubation time at each temperature must be monitored. Therefore, use of expensive automated equipment is mandatory when PCR is to be routinely employed in assays. Another problem is that, at best, the number of product molecules only doubles in each PCR cycle. However, even this low per cycle efficiency is rarely, if ever, met in practice. As a result, the number of cycles must be increased to reach a specific amplification level. Finally, PCR cannot distinguish RNA targets from DNA targets in mixed nucleic acid samples when the RNA and DNA sequences are colinear. Direct detection of RNA targets in such samples requires the removal of all DNA sequences.

Like all other amplification methods, PCR suffers several additional problems. First, not all primer sequences are equally efficient and the factors in choosing primer sequences are not fully known. Therefore, the utility of each primer pair must be determined experimentally. Further, the reaction conditions for each target and primer pair combination often need to be optimized. Second, extraneous sequences can be coamplified due to nonspecific hybridization of the primers. The severity of this problem increases with the level of amplification that is obtained. Third, false positives can result from contamination of reagents or equipment with the end-products of previous reactions. Such contamination represents the greatest problem in performing any target amplification reaction. Since PCR and other amplification reactions can generate more than 10⁶ copies of the target sequence starting from just one initial target molecule, accidental introduction of the end-product from previous reactions through aerosols or contact with common equipment easily leads to false positives, especially when high amplification levels are sought.

Amplification by RNA transcription (TAS, 3SR)

TAS was the first amplification system reported that was based on transcription.⁴ Figure 2 shows the basic

Table 2

Comparison between in vitro amplification systems

PCR	A. Advantages	LCR
Single enzyme reaction	Isothermal reaction	Single tube reaction
Single tube reaction	No instrumentation required	Single enzyme reaction
Thermostable enzyme available	Rapid reaction kinetics	Significant potential capability to combine amplification with detection of genetic mutations
Products have specific size	Single tube reaction	
Automated thermal cycling devices available	Product can be assayed by quantitative sandwich hybridization	
Reaction can be performed on crude target preparations	Product is single-stranded and can be sequenced directly	
Can be used on both RNA and DNA targets	Reaction has inherent ability to distinguish RNA from DNA targets	
Many special purpose modifications have been developed		
B. Disadvantages		
PCR	3SR	LCR
Many manual steps if no automated thermal cycling device available	Three-enzyme reaction	Enzyme efficiency is limited; long cycle times are required to reach two-fold amplification per cycle
Cycling requires time and temperature control	Lower specificity of amplification due to lower thermal stringency	High background problems due to nontarget directed ligation
Efficiency rarely equals the theoretical two-fold amplification per cycle	Lack of thermostable enzymes	Thermostable ligase not commercially available so manual thermal cycling required
Target preparation required to distinguish RNA from DNA in mixed nucleic acid samples	Larger oligonucleotide primers required	Requires four oligonucleotides

TAS protocol which involves generation of duplex cDNAs that contain the bacteriophage T7 transcription promoter followed by T7 RNA polymerase-mediated transcription of the cDNA to achieve amplification of the target sequence. Synthesis of the first strand of the cDNA is accomplished by primer extension of oligonucleotides that contained the polymerase binding sequence (PBS) at their 5'-end using Avian Myeloblastosis Virus (AMV) reverse transcriptase and target RNA (or denatured DNA) as template. After thermal denaturation of the RNA-DNA duplex, another primer is used to direct second strand synthesis of the cDNA. The duplex cDNA containing a promoter is then transcribed with T7 RNA polymerase to produce the antisense RNA amplification product. Further amplification of the target sequence can be accomplished by additional cycles of cDNA synthesis and transcription of the product RNA. In contrast to PCR, where at the most two copies of the target can be formed per cycle,

TAS produces between 10 and 100 copies of each target molecule per cycle. Therefore, a 10^6 -fold amplification of the target can be achieved in only 4-6 cycles (3-4 hr). The major drawback to the TAS protocol is the number of enzyme additions that must be made because of the lack of thermostable reverse transcriptase and RNA polymerase.

This problem has largely been circumvented in a modification of TAS protocol called 3SR, which uses enzymatic degradation of the RNA in the RNA-DNA heteroduplexes in place of thermal denaturation (*Figure 3*).⁵ Addition of RNase H to TAS protocol means that the reaction can be performed in a single tube, at a single temperature, and without reagent additions once the reaction is initiated. With 3SR, 10^6 - to 10^9 -fold amplification of target sequences has been achieved in a 1-hr incubation at 42 °C (Table 1). As with TAS, the majority of the amplification product is single-stranded RNA, which can have either the antisense sequence of

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TARGET AMPLIFICATION *continued*

TRANSCRIPTIONAL AMPLIFICATION SYSTEM (TAS)

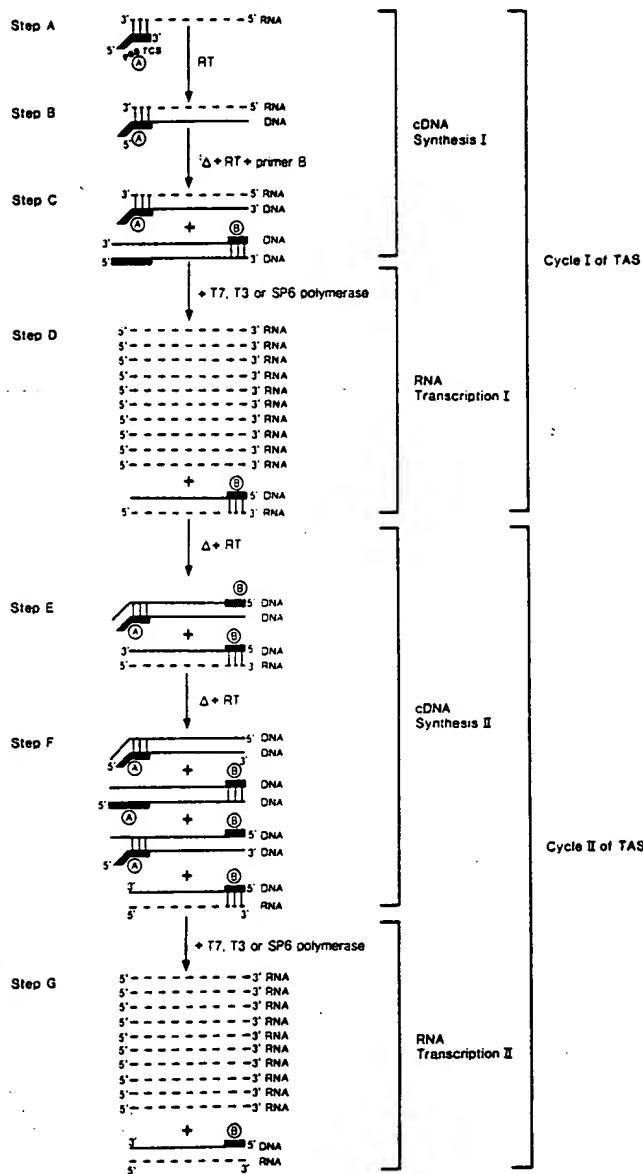


Figure 2 TAS—a two-cycle scheme for amplifying a RNA target sequence using sequential cDNA synthesis and RNA transcription is displayed as seven steps. The target RNA molecule (or denatured DNA) is hybridized to a primer oligonucleotide (primer A) that contains a T7 (or T3 or SP6) RNA polymerase binding site (PBS) and a target-complementary sequence (TCS) (step A). Reverse transcriptase (RT) elongates primer A to yield a newly synthesized DNA strand complementary to the target RNA (step B). The RNA-DNA heteroduplex is denatured by heat (Δ) and oligonucleotide B is annealed to the newly synthesized DNA strand containing the PBS. RT is added to produce a double-stranded cDNA and a new RNA-DNA heteroduplex (step C). Incubation of the double-stranded cDNA with T7 (T3 or SP6) RNA polymerase results in the synthesis of multiple RNA transcripts from the PBS-containing double-stranded DNA template (step D). Some of this RNA is immediately converted to RNA-DNA heteroduplex by RT (still present in the reaction mixture from step C) using oligonucleotide B as a primer. Further amplification of target sequences can be obtained by a second cycle of cDNA synthesis (steps E and F) and RNA transcription (step G).

3SR Amplification

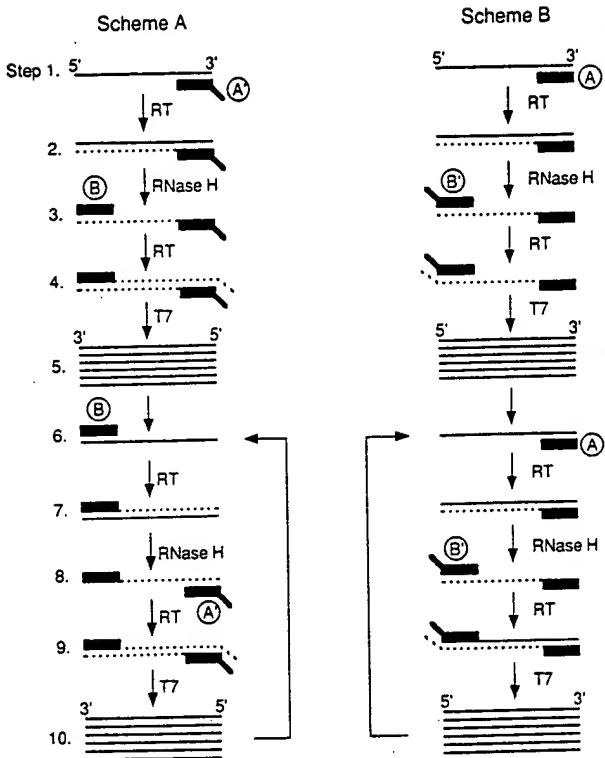


Figure 3 3SR—the 3SR reaction depends on continuous cycling of the reverse transcription and RNA transcription reactions to replicate an RNA target by means of cDNA intermediates. Pairs of oligonucleotides, A' and B or A and B', are used to prime cDNA synthesis. The tails on oligonucleotides A' and B' indicate that these primers contain the promoter sequence for the T7 RNA polymerase. Steps 1-4 depict the synthesis of a double-stranded cDNA which serves as transcription template for T7 RNA polymerase. Complete cDNA synthesis is dependent on the digestion of the RNA in the intermediate RNA-DNA heteroduplex (step 2) by RNase H. Transcription-competent cDNAs yield antisense (scheme A, step 5) or sense (scheme B, step 5) RNA copies of the original target. These transcripts are then converted to cDNAs containing double-stranded promoter sequences which can serve as template for further RNA synthesis.

the target (Figure 3, scheme A), the same sequences (scheme B), or both, depending on which primers have the T7 PBS. Approximately 1% of the amplification product exists as double-stranded cDNA and RNA-cDNA heteroduplex.

The 3SR system has a number of advantages over other amplification systems (Table 2a). First, the reaction is simple to perform. All reagents are pipetted into a single tube and the tube is incubated at a single temperature. No special thermal cycling equipment is required. Repeated reagent additions and tube transfers are also not required. In fact, for 3SR, the concept of cycle is only theoretical since this procedure has no manipulative cycles. Second, 3SR is rapid. A 10⁴-fold

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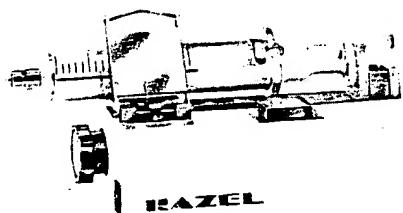
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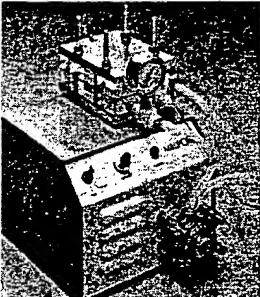
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TARGET AMPLIFICATION *continued*

amplification is achieved in 15 min and the reaction is complete ($\geq 10^6$ -fold amplification) in less than 1 hr. In contrast, the same level of amplification by either the PCR or TAS systems requires 3-4 hr.⁹ Third, the majority of the 3SR product is single-stranded RNA, which can be measured by a quantitative hybridization system. Hybridization systems, such as the bead-based sandwich hybridization system, do not perform well with the double-stranded DNA product produced by PCR.^{4,5} This single-stranded product can also be used directly for nucleic acid sequencing. Fourth, the method will specifically amplify RNA in mixed nucleic acid samples. Application of the 3SR system to DNA target sequences requires the use of thermal denaturation during the initial synthesis of cDNA incorporating the T7 promoter sequence. Without the use of these thermal denaturation steps, duplex DNA will not serve as a substrate for the 3SR reaction.

The disadvantages of the 3SR system (Table 2b) mainly revolve around the enzymes used in the reaction. Thermostable versions of these enzymes are not yet available and, therefore, the maximum incubation temperature for efficient amplification is only 42 °C. As a result, the amount of specific product produced in 3SR must be quantitated by hybridization since 3SR may produce significant levels of nonspecific RNA. Once thermostable enzymes become available and the incubation temperature of the reaction can be raised, the problem of nonspecific RNA should be mitigated.

Amplification by ligation (LAR/LAS)

DNA ligase can be used to amplify a target sequence through the repeated joining of oligonucleotides which hybridize to the target. This approach, which has been termed ligation amplification reaction (LAR)⁶ and ligase-based amplification system (LAS),⁷ uses four oligonucleotides, two per target strand (Figure 4). After hybridization of the two oligonucleotides to adjacent sequences on a target strand, the two oligonucleotides are joined by DNA ligase to form the product. The product is then separated from the target sequence by heat denaturation and both the ligation product and the target then serve as substrate for the next cycle of hybridization and ligation.

The greatest advantage of LAR/LAS is its potential to combine amplification with detection of genetic mutations (Table 2). Mispairing of the oligonucleotides to the target sequence at the bases to be joined will prevent ligation. As a result, success of the amplification will become dependent upon whether the probe oligonucleotides contain the normal or mutant sequence. This use of ligase has been incorporated into both a detection assay^{6,10} and a combined amplification-detection system⁶ for the sickle cell mutation in the β -globin gene.

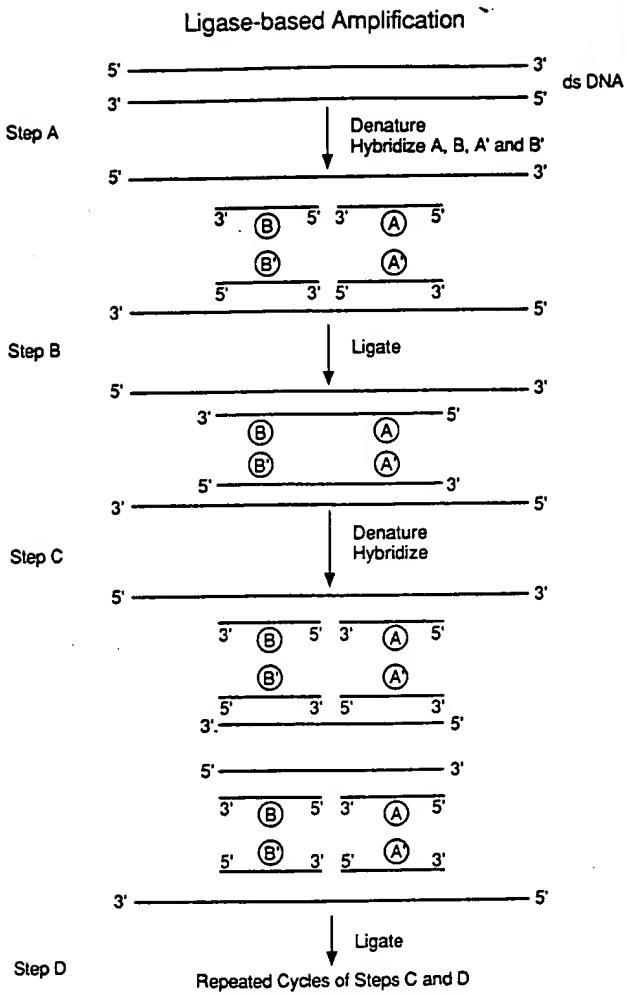


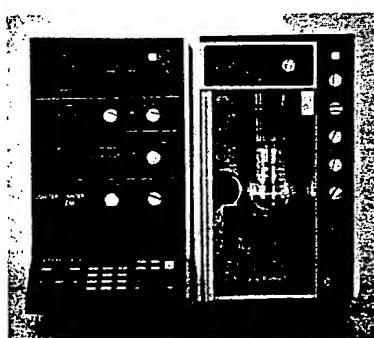
Figure 4 LAS—a double-stranded DNA target is thermally denatured in the presence of two sets of complementary oligonucleotides A/A' and B/B' where oligonucleotides A and B are complementary to adjacent sequences on the target DNA. The oligonucleotides A/B and A'/B' hybridize to opposite strands of the denatured DNA. A DNA ligase joins the 5'-ends of oligonucleotides B and A' to the 3'-ends of oligonucleotides A and B', respectively (step B). The ligated oligonucleotides are then thermally denatured from the target DNA. Oligonucleotides A/B and A'/B' are again hybridized to the appropriate target strands and to the ligated product strands (step C). These oligonucleotides are then joined by a DNA ligase (step D). Steps C and D are repeated to achieve exponential amplification of the ligated products AB and A'B'.

These ligase-based systems suffer two major drawbacks at present. The first problem is that the ligases which have been used, the *Escherichia coli* DNA ligase⁷ and the bacteriophage T4 DNA ligase,⁶ can give blunt-end ligation of duplex oligonucleotides as well as joining of single-stranded oligonucleotides. While the efficiency of these two activities is low, the large excess of free oligonucleotides relative to oligonucleotides hybridized to the target sequence in the ligation reaction can result in high background levels due to template-independent ligation. Reaction conditions which inhibit formation of template-independent product have been

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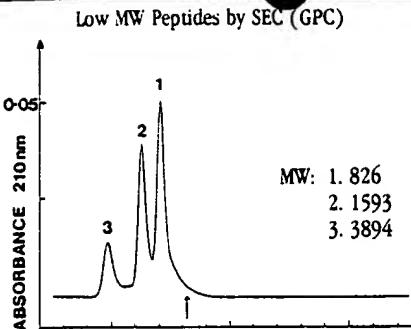
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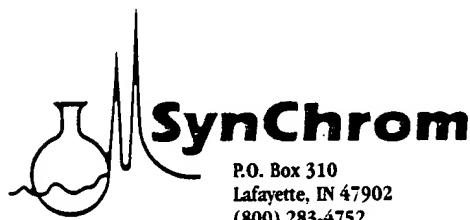
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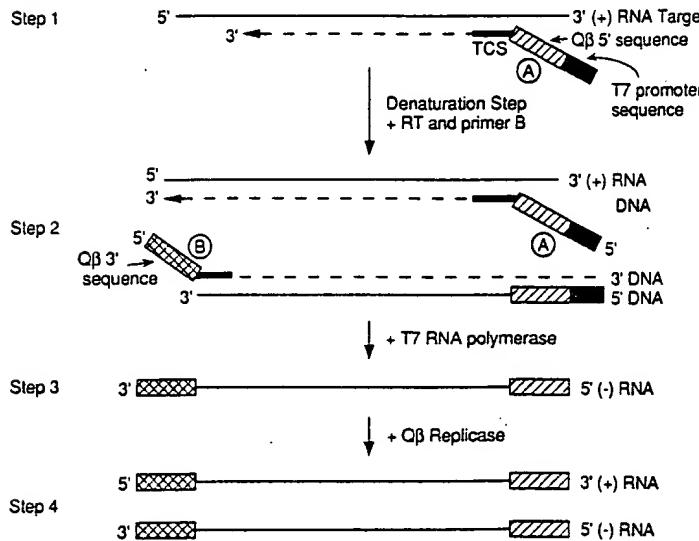
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TARGET AMPLIFICATION continued

Amplification by RNA Replication



found, but these lower the efficiency of the template-dependent ligase activity as well.⁶

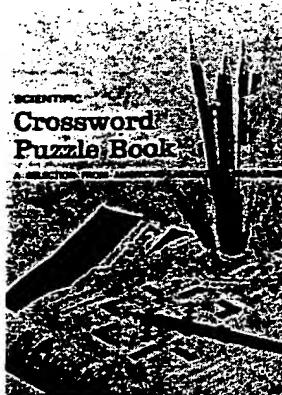
The second drawback is the limited amplification efficiency. Like PCR, each cycle of LAR/LAS should yield a two-fold amplification of the target sequence. However, kinetic properties of the ligase enzymes

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Figure 5 Amplification of RNA replication—the first two steps of amplification by RNA replication are identical to the first steps of TAS (Figure 2) except that oligonucleotide primer A contains the 5'-recognition sequence for Qβ replicase in between the T7 promoter sequence and target complementary sequence (TCS) and primer B contains the 3'-Qβ recognition sequence in addition to the TCS. Once the two primers have been incorporated into the cDNA copy, T7 RNA polymerase is used to transcribe the cDNA synthesizing antisense RNA copies of the target RNA. This RNA product contains the 5'- and 3'-Qβ recognition sequences at its ends (step 3). The Qβ replicase can then use this antisense RNA transcript as template for the synthesis of both sense and antisense RNA replication products.

makes this difficult to achieve, especially when genomic DNA is used as the target. In order to obtain 98% of the expected efficiency, Wu and Wallace⁶ had to incubate the reaction for 5 hr per cycle. The use of shorter cycle times results in a lower ligation efficiency and increases the number of cycles that must be per-

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formed.

Amplification by RNA replication ($\text{Q}\beta$)

The replicase for the RNA bacteriophage $\text{Q}\beta$ has been used for amplification of hybridization signal.⁸ For signal amplification, a recombinant probe RNA sequence containing both the required 5'- and 3'-replicase recognition sequences was hybridized to immobilized target sequences. After extensive washing to remove unhybridized probe RNA, the probe RNA was eluted from the target sequence and incubated with the $\text{Q}\beta$ replicase. A 10⁷-fold amplification of the probe sequence was observed in a 20-min incubation. However, significant background problems were also encountered. Any small amount of probe RNA that is nonspecifically retained also becomes highly amplified due to the high efficiency of the $\text{Q}\beta$ replicase.

The $\text{Q}\beta$ replicase should theoretically be useful in target amplification as well. In the protocol shown in *Figure 5*, substrate RNA for the replicase would be synthesized by performing one cycle of the TAS protocol using primers which contain the $\text{Q}\beta$ replicase 5'- and 3'-recognition sequences. The TAS product would then be further amplified by replication of the RNA with the $\text{Q}\beta$ replicase. Such a system should have the potential to produce a 10⁶- to 10⁹-fold amplification of a target sequence in 1 hr, allowing 30 min for the one cycle of TAS and 30 min for $\text{Q}\beta$ replication (Table 1). Since formation of the replicase substrate in this protocol is dependent on successful synthesis of the

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TAS product, background problems should be much less than in the signal amplification system.

Summary

Currently, PCR is the standard method for target amplification because it is the oldest and most developed procedure. However, several new alternative approaches for target amplification have recently been developed. Although these new methods are at a relatively early stage of development, each has some advantages over PCR, such as greater amplification per cycle (TAS, 3SR, $\text{Q}\beta$), isothermal reaction (3SR), or coupled amplification-mutation detection (LAR/LAS). As a result, each may eventually gain widespread use after further development.

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